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this Application:

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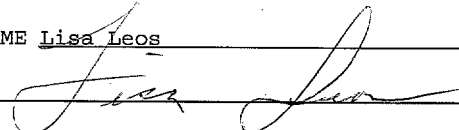
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Sir:

This is a request for filing an

☐ Original

☒ Continuation

☐ Divisional

☐ Continuation-in-part

application under 37 C.F.R. 1.53(b), in the name of Henry A. Lester; Norman Davidson; Paulo Kofuji (Names of ALL Applicants), for INWARD RECTIFIER, G-PROTEIN ACTIVATED, MAMMALIAN, POTASSIUM CHANNELS AND USES THEREOF (Title of Invention). This ☒ continuation ☐ divisional ☐ continuation-in-part claims priority to pending application Serial No. 08/614,801, filed on March 7, 1996.

1. (a) ☐ Enclosed is a new application.
- (b) ☐ Enclosed is a continuation-in-part application.
- (c) ☒ Enclosed is a copy of the prior application.

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Form 1.16b (8069)

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2. (a) ☐ Enclosed is a new Declaration.
 (b) ☒ Enclosed is a copy of the prior executed Declaration as originally filed.
 (c) ☐ Enclosed is a Combined Declaration/Power of Attorney.
3. (a) ☐ Enclosed is a Small Entity Affidavit.
 (b) ☒ A Small Entity Affidavit is of record in the prior application.
4. ☒ The filing fee is calculated below:

Claims as filed in the prior application, less any claims canceled by amendment below:

FOR:	(Col. 1)	(Col. 2)	SMALL ENTITY		OR	OTHER THAN A SMALL ENTITY	
	NO. FILED	NO. EXTRA	RATE	FEE		RATE	FEE
BASIC FEE				\$395	OR		\$790
TOTAL CLAIMS	<u>17</u>	-20 = * <u>0</u>	x11 =	\$ <u> </u>	OR	x22 =	\$ <u> </u>
INDEP CLAIMS	<u>4</u>	-3 = * <u>1</u>	x41 =	\$ <u>41</u>	OR	x82 =	\$ <u> </u>
[] MULTIPLE DEPENDENT CLAIM PRESENTED			+135 =	\$ <u> </u>	OR	+270 =	\$ <u> </u>
*If the difference in Col. 1 is less than zero, enter "0" in Col. 2.			TOTAL	\$ <u>436</u>	OR TOTAL		\$ <u> </u>

5. ☒ The Filing Fee is not enclosed.
6. ☐ Our check in the amount of \$ is enclosed.
7. ☐ Cancel in this application original claims of the prior application before calculating the filing fee. (At least one independent claim must be retained for filing purposes.)
8. ☒ Amend the specification by inserting before the first line the sentence:

--This is a ☒ continuation ☐ division ☐ continuation-in-part of application Serial No. 08/614,801 filed March 7, 1996.--

15. _____ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)
16. _____ A Prior Art Statement is enclosed.
17. X I hereby verify that the attached papers are a true duplicate of prior application Serial No. 08/614,801 as originally filed on March 7, 1996.

Date: 3-14-98 

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INWARD RECTIFIER, G-PROTEIN ACTIVATED, MAMMALIAN, POTASSIUM CHANNELS AND USES THEREOF

The invention disclosed herein was made with U.S. Government support
10 under USPHS grants GM29836 and MH49176. The U.S. government has
certain rights in this invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application Serial
15 no. 08/066,371, filed March 21, 1993.

INTRODUCTION

Background

The regulation of heart rate in response to stress, activity, and other
20 stimuli is essential for mammalian survival. While each independent heart
muscle has an inherent faculty for independent excitation, the heartbeat is
initiated in a specialized group of muscle cells in the sinoatrial node of the
heart, which form the pacemaker. The heart is also innervated by nerves that
regulate the beat. When these nerves are active, they liberate chemicals such
25 as noradrenaline or acetylcholine from their terminals, and these
neurotransmitters affect the cardiac muscles directly. The pacemaker is
inhibited by acetylcholine and excited by noradrenaline.

The release of acetylcholine (ACh) opens a K^+ channel in the atrium,
slowing the rate of depolarization that leads to initiation of the action potential.

This effect is mediated through a G-protein signal transduction pathway, involving a pertussis toxin-sensitive, heterotrimeric G-protein, G_K , probably belonging to the G_i/G_o family. Activation of the K^+ channel by G_K does not require cytoplasmic intermediates, suggesting direct coupling of one or more
 5 G-protein subunits to the channel. However, a long-standing controversy exists as to which subunit couples to the channel. Both purified $\beta\gamma$ subunit complex and α subunits of the G_i/G_o family activate the channel in cell free, inside-out patches of atrial myocytes. Activation by the α subunits occurs at lower concentrations than that by $\beta\gamma$, but seems to be less efficient. The relative
 10 physiological importance of each pathway, as well as of possible involvement of the arachidonic acid pathway, is unclear.

A similar K^+ channel is activated in the atrium by adenosine, ATP and epinephrine, probably also via a G-protein pathway. Furthermore, in nerve cells various 7-helix receptors, such as serotonin 5HT1A, δ -opioid, $GABA_B$ and
 15 somatostatin couple to similar K^+ channels, probably through direct activation by G-proteins. The similarity of the channels and signaling pathways in atrium and nerve cells is also shown by the coupling of a neuronal 5HT1A receptor (5HT1A-R) to the atrial channel, through transient expression in myocytes.

By electrophysiological and pharmacological criteria, these K^+ channels
 20 belongs to a family of inward rectifiers that conduct K^+ much better in the inward than the outward direction, are blocked by extracellular Cs^+ and Ba^{2+} , and are believed to possess a single-file pore with several permeant and blocking ion binding sites. Recently, the primary structures of two mammalian inward rectifier channels have been elucidated by cDNA cloning: an ATP-
 25 regulated K^+ channel from kidney, and an inward rectifier from a macrophage cell line. Both appear to belong to a new superfamily of K^+ channels, with only two transmembrane domains per subunit and a pore region homologous to that of K^+ , Ca^{2+} and Na^+ voltage-dependent channels.

G-protein regulated K⁺ channels are important for the regulation of heart and nerve function. Determination of their molecular structure and regulation is therefore of great interest. Cloning of the channel protein genes and expression in a heterologous system would allow a molecular approach to investigation and manipulation of these regulatory pathways.

Relevant Literature

The activation of atrial K⁺ channels by G-proteins is reviewed in Kurachi *et al.* (1992) Prog. Neurobiol. **39**:229-246; and Brown and Birnbaumer (1990) A. Rev. Physiol. **52**:197-213. Logothetis *et al.* (1987) Nature **325**:321-326 and Kurachi *et al.* (1989) Pflugers Arch. **413**:325-327 provide evidence for activation by the $\beta\gamma$ subunits. Codina *et al.* (1987) Science **236**:442-445 show activation by the α -subunit. Karschin *et al.* (1991) P.N.A.S. **88**:5694-5698 demonstrates the coupling of a neuronal receptor to the atrial K⁺ channel.

Physiological characterization of the atrial K⁺ channels is reviewed by Hille, B. (1992) *Ionic Channels of Excitable Membranes*, 2nd edition (Sinauer, Sunderland, Mass). The role of Mg²⁺ in blocking K⁺ efflux is discussed in Horie and Irisawa (1987) Am. J. Physiol. **253**:H210-H214.

The sequence characterization of a mammalian inward rectifier K⁺ channel is disclosed in Ho *et al.* (1993) Nature **362**:31-38; and Kubo *et al.* (1993) Nature **362**:127-132. A brief review of these inward rectifying K⁺ channels may be found in Aldrich (1993) Nature **362**:107-108.

SUMMARY OF THE INVENTION

Compositions and methods are provided for producing functional mammalian inward rectifier, G-protein activated potassium channels (Kir3.0 channels). The functional channels comprise one or more polypeptides from the Kir3.0 subfamily of inward rectifier potassium channels. The effect that

G-protein subunits exert on channel activity is determined by the specific composition of polypeptides in the channel. The functional Kir3.0 channels are useful in drug screening assays directed to modulation of cellular electrophysiology. Nucleic acids encoding Kir3.0 polypeptides are useful for expression of the gene product, and for identification of homologous genes from other species, as well as other members of the same family of proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows inward currents evoked by high K^+ , 5HT and ACh in RNA-injected oocytes. (A) I_{hK} and I_{5HT} in an oocyte injected with atrial RNA + 5HT1A-R RNA. Holding potential in this and all following figures was -80mV. (B) Inward currents evoked by ACh (AcChO) and 5HT in a single oocyte in hK solution. (C) The dependence of I_{5HT} amplitude on 5HT concentration in oocytes of one frog. In each oocyte, the response to one 5HT concentration was tested. Data represent mean \pm SEM in 4-6 cells at each concentration.

Figure 2. I_{hK} and I_{5HT} are inwardly rectifying K^+ currents. (A) Currents evoked by voltage steps from the holding potential of -80 mV to voltages between -140 and 40 mV in 20 mV steps in ND96(a), hK (b), hK in the presence of 5HT (c). Net I_{5HT} (d) was obtained by digital subtraction of (b) from (c). (B) Current-voltage relations of the total membrane current in a representative oocyte in NG 96 (2 mM $[K_{out}]$; \square), in 25 mM $[K^+_{out}]$ \blacklozenge ; in 75 mM $[K_{out}]$ \circ , and in hK (96 mM $[K_{out}]$; \blacktriangle). (C) Current-voltage relation of the net I_{5HT} in the same oocyte as in (B) in 25 mM $[K_{out}]$ \blacklozenge , 75 mM $[K_{out}]$ \circ , and 96 mM $[K_{out}]$ \blacktriangle . (D) The dependence of the reversal potentials of total membrane current \blacktriangle and of I_{5HT} \bullet on $[K_{out}]$. The straight lines represent least square fits to data (mean \pm SEM, n=3 for each point).

Figure 3. Ba^{2+} block of I_{hK} and I_{5HT} . (A-C), records taken from the same oocyte at 10 min intervals. Between the records, the cell was bathed in ND96.

5HT concentration was 4 nM. Note that in (B) 300 μ M Ba²⁺ reduces I_{hK} and almost completely blocks I_{5HT}. Ba²⁺ and 5HT were washed out simultaneously, and this resulted in an inward current "tail". (D) dose dependence of Ba²⁺ inhibition of I_{hK} in native oocytes ○, I_{hK} in RNA-injected oocytes ●, I_{5HT} in RNA-injected oocytes ▽. Data are mean±SEM, n=3 to 7 for each point.

Figure 4. I_{5HT} is mediated by activation of a G-protein. (A) The effect of PTX treatment (500 ng/ml, 20-26 h) on I_{hK} and I_{5HT}. The cells were injected with 120 ng/oocyte total atrial RNA, 11 ng/oocyte 5HT1A-R RNA, and, where indicated, with 11 ng/oocyte G_{i2}α RNA. (B) GDP-β-S injection inhibits I_{5HT} but not I_{hK} in an oocyte injected with atrial + 5HT1A-R RNAs. 5HT concentration was 0.4 μ M. A small outward current deflection (denoted by ★) upon washout of 5HT was caused by an inadvertent perfusion of ND96 for a few seconds.

15 DATABASE REFERENCES AND NOMENCLATURE FOR SEQUENCES

Systematic nomenclature for the family of inward rectifying potassium channel proteins has been proposed by Doupnik *et al.* (1995) Curr. Opin. Neur. 5:268-277. This family is characterized by the presence of only two transmembrane domains per subunit, and a pore region homologous to that of K⁺, Ca²⁺ and Na⁺ voltage-dependent channels. As suggested by Doupnik *et al.*, the G-protein regulated members of this family are grouped into the Kir3 subfamily, where "Kir3.0" designates the subfamily of genes and polypeptides, and Kir3.1, 3.2, etc. refer to the specific subfamily members. Other subfamilies include the ATP regulated channels predominantly expressed in the kidney (Kir1.0 subfamily), and the constitutively active, steep inward rectifying Kir2.0 subfamily.

the polypeptides may be from the same or different species. The functional channel has the distinctive features of an inward rectifier, in that it conducts inward but not outward K^+ current; it is blocked by low concentrations of extracellular Cs^+ or Ba^{2+} ; and the conductance of the channel does not depend solely on voltage, but on $(E-E_K)$. Characteristic of the Kir3.0 family, the ability of the channel to conduct K^+ current is modulated by G-proteins, particularly G-proteins of the G_i/G_o family. It has been found that such modulation may be mediated directly by the G-protein $\beta\gamma$ subunit. It will be understood by one of skill in the art that a number of mammalian cell surface receptors activate G-proteins as a consequence of specific ligand binding. The signal transduction from receptor to Kir3.0 channel can therefore be coupled through G-protein intermediates.

The specific combination of Kir3.0 polypeptides in the channel will determine the properties of the channel. As an example, it has been observed that a channel comprised of Kir3.1 and Kir3.2 polypeptides, or of Kir3.1 and Kir3.3 polypeptides, demonstrates a significant enhancement of the G-protein evoked currents. In contrast, a channel comprised of Kir3.2 and Kir3.3 polypeptides shows a decrease in G-protein evoked currents.

As used herein, the term Kir3.0 polypeptide is used to designate a single polypeptide that is capable of associating with other Kir3.0 polypeptides to form a functional Kir3.0 channel as defined above. When a nucleic acid encoding a Kir3.0 polypeptide is introduced into a cell, particularly a *Xenopus* oocyte, and expressed, it confers a change in the electrophysiology of the cell, such that the cell has an altered ability to conduct K⁺ current, particularly in response to G-protein activation. Genes encoding Kir3.0 polypeptides can be identified in such a system based on the expression of a single gene, or a combination of genes. The ability of Kir3.0 polypeptides to associate and form multimeric channels in a cell provides a means of identifying genes encoding

novel Kir3.0 polypeptides, where a candidate gene may be introduced into a cell comprising a known Kir3.0 gene. A change in the electrophysiology of the cell, as described above, is indicative that the candidate gene encodes a Kir3.0 polypeptide able to partake in multimeric channel formation.

- 5 Kir3.0 polypeptides may also be characterized by sequence similarity to known members of the Kir3.0 family. SEQ ID NO:2 describes an exemplary Kir3.1 polypeptide, of rat origin. Within a species, various members of the Kir3.0 gene family will usually have at least about 50% amino acid sequence identity, more usually at least about 60%, and may be as high as 70 to 80%.
- 10 Between mammalian species, the homologous Kir3.0 polypeptides, e.g. human and rat Kir3.1, etc., have a high degree of similarity, usually at least about 75% amino acid sequence identity, more usually at least about 85% sequence identity, and may be as high as 90% sequence identity, particularly in the conserved transmembrane and pore forming regions.
- 15 For convenience in experimental manipulation and drug screening assays, the Kir3.0 channel may be assembled in an expression host cell. As used herein, an expression host cell is a cell, preferably a eukaryotic cell having substantially no endogenous Kir3.0 channels. The expression host will have introduced into it one or more different exogenous nucleic acid(s)
- 20 encoding one or more Kir3.0 polypeptides, preferable at least two polypeptides. Such exogenous nucleic acids will be capable of constitutive or inducible expression of the Kir3.0 nucleic acid(s) in the host cell. Where two or more polypeptides are present, the genes encoding Kir3.0 polypeptides may be on a single vector or DNA molecule, or may be present on separate molecules. The
- 25 expression of the exogenous nucleic acid causes an altered ability to conduct K^+ current in the expression host cell, particularly in response to G-protein activation. For example, nucleic acids encoding one or more mammalian Kir3.0 polypeptides can be inserted into a host cell, e.g. *Xenopus* oocyte; yeast;

mammalian immortalized cell line or primary cell culture; plant cell; *etc.* by transfection, injection, transduction, *etc.* Expression of the nucleic acid produces Kir3.0 polypeptides, which will assemble into a channel in the membrane of the host cell. It has been found in some cases that the
 5 heterologous Kir3.0 polypeptide will assemble with polypeptides of the heterologous host cell to form a hybrid channel. Cell surface receptors, such as muscarinic receptors, serotonin receptors, *etc.*, and G-proteins may additionally be introduced into the heterologous host cell. A functional heterologous channel will regulate inward K^+ current in response to the
 10 presence of exogenous or endogenous activated G-proteins.

The Kir3.0 channels in homologous or heterologous cells are useful in the study of cell electrophysiology, including but not limited to neural and cardiac cells, particularly G protein linked responses, such as those coupled to acetylcholine and serotonin receptor binding. Of particular interest are *in vitro*
 15 drug screening assays. Screening assays are provided to identify drug candidates that specifically alter Kir3.0 channel activity either directly or indirectly by modulating receptors, G-proteins, channels or the interactions among these elements. Novel binding agents include specific antibodies, natural or non-natural binding agents identified in screens of chemical
 20 libraries, analogs of acetylcholine, G proteins, *etc.* Areas of investigation include the development of cardiovascular, neurologic endocrine and gastrointestinal treatments.

Nucleic acid molecules are provided that encode individual polypeptides capable of forming a Kir3.0 channel. The subject polypeptide may be any one
 25 of Kir3.1 (for purposes of continuity referred to hereafter as Kir3.1/KGA), Kir3.2, Kir3.3, Kir3.4, *etc.* The origin of the nucleic acid may be any mammalian species, e.g. human, murine, primate, bovine, equine, canine, feline, ovine, porcine, *etc.*

The nucleic acid compositions of the subject invention encode all or a part of the subject Kir3.0 polypeptides. Fragments may be obtained of the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, etc. For the most part, DNA fragments will be of at least 15 nt, more usually at least 18 nt. Preferably fragments will encode a functional epitope or domain. The sequence providing for a functional epitope can be determined by expression of the sequence, and assaying for reactivity of the expression product with specific antibodies by conventional immunoassay, or by assaying for the ability of the fragment to create a polypeptide capable of assembling into a functional Kir3.0 channel.

The DNA sequences may be obtained in substantial purity, and will be obtained as a sequence other than a sequence of an intact chromosome. Usually, the DNA will be obtained substantially free of other nucleic acid compounds which do not include a Kir3.0 sequence or fragment thereof, generally being at **least** about 50%, usually at least about 90% pure and are typically "recombinant", *i.e.* flanked by one or more nucleotides with which they are not normally associated with on a natural chromosome.

The subject nucleic acids may be used in a variety of ways. They may be used as probes for identifying other Kir3.0 genes. Homologous sequences and related members of the Kir3.0 gene family are those with substantial sequence similarity to the subject sequences, as previously described.

- 5 Algorithms for sequence analysis are known in the art, and include BLAST, described in Altschul *et al.* (1990) J Mol Biol **215**:403-10; ADVANCE and ADAM, described in Torelli and Robotti (1994) Comput Appl Biosci **10**:3-5; and FASTA, described in Pearson and Lipman (1988) P.N.A.S. **85**:2444-8.

- Such homologous or related nucleic acid sequences are detected by
- 10 hybridization under low stringency conditions, for example, at 50° C and 10XSSC (0.9 M saline/0.09 M sodium citrate) and remain bound when subject to washing at 55°C with 1XSSC. The high degree of similarity between Kir3.0 homologs allows the use of relatively stringent hybridization conditions, as known in the art. For example, see Sambrook *et al. Molecular Cloning* (Cold
- 15 Spring Harbor, New York, 1989). By using probes, particularly labeled probes of DNA sequences, one may be able to isolate homologous genes, which may be then used for identifying members of the Kir3.0 family in other species. By probes is intended a single stranded oligonucleotide of at least about 12 nt in length, more usually at least about 15 nt, and preferably at least about 18 nt,
- 20 characterized by the ability to hybridize under low stringency conditions to Kir3.0 genes. The term "unique" as used herein defines a nucleic acid molecule that does not contain known genomic repeated sequences, including but not limited to *Alu* sequences.

- Various methods are known in the art for identification of nucleic acids
- 25 having sequence similarity, based on the ability of single stranded nucleic acids, e.g. DNA to form a double stranded complex. A sample may be screened for the presence of nucleic acids capable of forming double stranded complexes with a Kir3.0 probe, particularly under low stringency conditions.

"Sample" as used herein includes but is not limited to genomic libraries, cDNA libraries, nucleic acid molecule extracts from tissue, or nucleic acid molecule extracts from cell culture. After complex formation, the other nucleic acid molecule is isolated by methods known in the art. Methods include the
5 polymerase chain reaction (PCR) or reverse transcriptase PCR (RT-PCR), where two probes from the subject nucleic acids sequence are used to amplify a region of the sample DNA or cDNA. PCR may also be performed with a single Kir3.0 probe, and a second oligonucleotide complementary to a known genomic repeat sequence. Alternatively, northern or southern blots are useful
10 in identifying bands, colonies, plaques, *etc.* comprising nucleic acids capable of hybridizing to a Kir3.0 probe. Generally the Kir3.0 probe will be labeled with a detectable marker, *e.g.* ^{32}P , ^{35}S , biotin, FITC, *etc.* when used in a northern or southern blot.

The subject nucleic acids may also be used to identify the expression of
15 Kir3.0 genes in a biological specimen. The manner in which one probes cells for the presence of particular nucleotide sequences, particularly as genomic DNA, is well-established in the literature and does not require elaboration here. Conveniently, a biological specimen is used as a source of mRNA. The mRNA may be amplified by polymerase chain reaction using primers specific for the
20 subject DNA sequences. Alternatively, the mRNA sample is separated on gel electrophoresis and then probed using Northern blotting with a fragment of the subject DNA as a probe. Other techniques may also find use. Detection of mRNA having the subject sequence is indicative of Kir3.0 expression in the sample.

25 The subject nucleic acid sequences may be modified for a number of purposes, particularly where they will be used intracellularly, for example, by being joined to a nucleic acid cleaving agent, *e.g.* a chelated metal ion, such as iron or chromium for cleavage of the gene; as an antisense sequence; or the

like. Modifications may include replacing oxygen of the phosphate esters with sulfur or nitrogen, replacing the phosphate with carboxamide, phosphoramidate, etc.

5 The DNA sequence may encode amino acid sequences that differ from the native sequence of a Kir3.0 polypeptide, but that do not produce functional changes in the Kir3.0 polypeptide. The sequence may encode polypeptide analogs, fragments or derivatives of substantially similar polypeptides that differ from the naturally-occurring forms in terms of the identity of location of one or more amino acid residues (deletion analogs containing less than all of the
10 residues specified for the protein, substitution analogs wherein one or more residues are replaced by other residues and addition analog wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These sequences include the incorporation of preferred codons for
15 expression in non-mammalian host cells; the provision of sites for cleavage by restriction endonuclease enzymes; the addition of promoters operatively linked to enhance RNA transcription; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

20 For expression, the DNA sequences may be inserted into an appropriate expression vector, where the native transcriptional initiation region may be employed or an exogenous transcriptional initiation region, *i.e.* a promoter other than the promoter which is associated with the gene in the normally occurring chromosome. The promoter may be introduced by recombinant
25 methods *in vitro*, or as the result of homologous integration of the sequence into a chromosome. A wide variety of transcriptional initiation regions are known for a wide variety of expression hosts, where the expression hosts may involve prokaryotes or eukaryotes, particularly *E. coli*; *B. subtilis*; yeast cells;

mammalian cells; e.g. Cos cells, HeLa cells, L(tk-), primary cultures; insect cells; *Xenopus laevis* oocytes; and the like. Generally a selectable marker operative in the expression host will be present. The promoter may be operably linked to the coding sequence of the genes of interest so as to produce a translatable mRNA transcript. Expression vectors have convenient restriction sites located near the promoter sequence so as to provide for the insertion of nucleic acid sequences encoding heterologous proteins. The promoters in suitable expression vectors may be either constitutive or inducible. Expression vectors for the production of fusion proteins, where the exogenous fusion peptide provides additional functionality, i.e. increased protein synthesis, stability, reactivity with defined antisera, an enzyme marker, e.g. β -galactosidase, etc.

Expression vectors may also be used to produce mRNA encoding a Kir3.0 polypeptide. The mRNA may be isolated, and used for synthesis of the polypeptide by injecting the RNA molecules into *Xenopus* oocytes and culturing the oocytes under conditions that are well known to an ordinary artisan.

The subject nucleic acids are used to identify nucleic acids encoding receptors and G-proteins that modulate the activity of Kir3.0 channels by activating or deactivating the channel. As previously described, such methods may also be used to identify genes encoding novel Kir3.0 polypeptides as well. A candidate nucleic acid or library of nucleic acids may be introduced into an expression host cell comprising a Kir3.0 channel, and the host cell screened for a change in the Kir3.0 channel activity. The candidate nucleic acid or library of nucleic acids may be selected from a variety of sources, including cDNA expression constructs operable in the host cell or mRNA transcribed *in vivo* or *in vitro* and subsequently introduced into the host cells. Pools of different nucleic acids may be tested, where positive pools are then used to generate smaller pools or individual clones. Nucleic acids that give a positive signal for

modulation of Kir3.0 channel activity are then isolated and characterized as to the nucleic acid sequence and structure. The change in channel activity may be the result of expression of the candidate nucleic acid directly, or a known activating agent may be added to the cells to effect a change in channel activity.

- 5 A candidate nucleic acid encoding a putative receptor or G protein that interacts with the subject channels may be tested for its ability to modulate Kir3.0 channel activity. The candidate nucleic acid is introduced into an expression host in conjunction with a Kir3.0 expression construct of one or more Kir3.0 polypeptides, preferably at least two polypeptides. The expression
- 10 host is contacted with a known G-protein associated receptor activator, where an increase in Kir3.0 channel conductance indicates activation of the channel, and a decrease in conductance indicated inhibition of the channel.

- Expression cassettes may be prepared comprising a transcription initiation region, which may be constitutive or inducible, the gene encoding the
- 15 subject methyltransferase or fragment thereof, and a transcriptional termination region. Of particular interest is the use of sequences which allow for the expression of functional epitopes or domains, usually at least about 24 nucleotides in length, more usually at least about 48 nucleotides in length, and up to the complete open reading frame of the gene. After introduction of the
- 20 DNA, the cells containing the construct may be selected by means of a selectable marker, the cells expanded and then used for expression.

- The expression cassettes may be introduced into a variety of vectors, where the vectors will normally be characterized by the ability to provide selection of cells comprising the expression vectors. The vectors may provide
- 25 for extrachromosomal maintenance, particularly as plasmids or viruses, or for integration into the host chromosome. Where extrachromosomal maintenance is desired, an origin sequence will be provided for the replication of the plasmid, which may be a low- or high-copy plasmid. A wide variety of markers

are available for selection, particularly those which protect against toxins, more particularly against antibiotics. The particular marker that is chosen will be selected in accordance with the nature of the host, where in some cases, complementation may be employed with auxotrophic hosts, e.g. bacteria, yeast.

- 5 Introduction of the DNA construct may be by any convenient means, e.g. conjugation, calcium-precipitated DNA, electroporation, fusion, transfection, infection with viral vectors, *etc.*

- 10 With the availability of the protein in large amounts by employing an expression host, the protein may be isolated and purified in accordance with conventional ways. A lysate may be prepared of the expression host and the lysate purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. The purified protein will generally be at least about 80% pure, preferably at least about 90% pure, and may be up to 100% pure. By pure is intended free of other proteins, as well as
- 15 cellular debris.

- The polypeptide may be used for the production of antibodies. Antibodies may be prepared in accordance with conventional ways, where the expressed polypeptide or protein is used as an immunogen, by itself or conjugated to known immunogenic carriers, e.g. KLH, BSA, *etc.* Various
- 20 adjuvants may be employed, as appropriate. For monoclonal antibodies, after one or more booster injections, the spleen of the immunized animal is isolated, the splenocytes immortalized, and then screened for high affinity antibody binding. The immortalized cells, *i.e.* hybridomas, producing the desired antibodies may then be expanded. For further description, see
- 25 Monoclonal Antibodies: A Laboratory Manual, Harlow and Lane eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 1988. The antibodies may find use in diagnostic assays for detection of the presence of Kir3.0 channels in patient samples.

By providing for the production of large amounts of Kir3.0 polypeptides and channels, one can identify ligands or substrates which bind to, or modulate the action of Kir3.0 channels. The subject Kir3.0 channels, polypeptides or functional domains thereof are used to screen for agonists or antagonists that modulate the K⁺ channel activity, by increasing or decreasing the evoked K⁺ current. In this way, drugs may be identified that can alter the electrophysiology of cells comprising Kir3.0 channels, including atrial, neural, vascular smooth muscle, endothelial, pancreatic and other cells. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including whole cell or single channel current quantitation in response to stimulus, patch clamping, labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like.

In a preferred embodiment, a whole cell or single channel current will be quantitated in the presence of a candidate pharmacological agent. The assay will include contacting a cell into which has been introduced a functional Kir3.0 channel with a candidate pharmacological agent, and detecting any change in channel conductance. An increase in inward K^+ channel conductance indicates channel activation, while a decrease indicates that the agent is a channel inhibitor.

The term "agent" as used herein describes any molecule, protein, or pharmaceutical with the capability of directly or indirectly altering ion channel conductance by affecting second messenger systems, receptors, G-proteins, interactions among the elements, or the ion channel directly. Agents include but are not limited to serotonin, neurotrophin, enkephalins, dopamine, arachidonic acid, cholera toxin, and pertussis toxin. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these

concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

The term "activators" as used herein defines any agent which activates a Kir3.0 channel, G-protein associated receptor or G-protein. The term "activates" as used herein is applied to both G-protein associated receptors and ion channel conductance and in terms of G-protein associated receptors defines the state of the receptor wherein it initiates release of a G-protein subunit which in turn initiates a cellular response. In terms of the ion channel conductance "activates" defines the state of the channel wherein the channel increases conductance. The term "deactivates" as used herein defines the state of the channel wherein the channel is initiated to decrease conductance or is incapable of conductance under conditions when the channel normally conducts ions across a membrane. The term "agonist" as used herein defines an agent that initiates activation of ion channel conductance or initiates activation of a second messenger system. The term "antagonist" as used herein defines an agent that initiates deactivation of ion channel conductance or initiates deactivation of a second messenger system.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon, heterocyclic or carbocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides,

saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

Various techniques for performing screening assays are known in the art. For example, a screening assay may utilize one or more molecules conjugated to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like. A mixture of the components are added to the assay sample, and the binding of candidate agents to the subject channels, receptors or G proteins is detected. Alternatively, a host cell expressing a Kir3.0 channel is combined with a candidate agent under conditions that induce a change in K⁺ current.

When an agent is identified that has a desired pharmacological activity, it may be used for prophylactic, therapeutic or experimental purposes. Such agents find use in investigating the interactions of inward K⁺ channels with surface membrane receptors, G-proteins and second messenger systems. For therapeutic use, the agent may be administered in a physiologically

acceptable carrier to a host. The agents may be administered in a variety of ways, orally, parenterally *e.g.* subcutaneously, intraperitoneally, intravascularly, intralingually, topically, *etc.* Depending upon the manner of introduction, the compounds may be formulated in a variety of ways, where the concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%. 5

For convenience, a kit may be provided having the components necessary to perform the subject screening assays. Such a kit may contain at least one expression vector comprising a Kir3.0 gene. Usually at least two Kir3.0 genes will be included, which may be on one vector or two vectors. The kit may further comprise agents for the detection of K⁺ current change, and agents having a known positive or negative effect on activity of Kir3.0 channels or receptors associated therewith. 10

The following examples are offered by way of illustration and not by way of limitation. 15

EXPERIMENTAL

Isolation of a cDNA encoding Kir3.1/KGA

A cDNA plasmid library was made from 19-day-old rat atrial mRNA. The library was linearized and amplified by polymerase chain reaction using primers complementary to sequences flanking the cDNA insert. cRNA was synthesized *in vitro* from the T7 promoter and microinjected into *Xenopus laevis* oocytes. Electrophysiological recordings identified an inward rectifier, G-protein activated, potassium channel. 20 25

Materials and Methods

Preparation of RNA and oocytes. Total RNA was extracted from atria and ventricles of 19-21 day old rats of both sexes using the procedure of

Chomczynski and Sacchi (1987) Anal. Biochem. **162**:156-159. Poly (A) RNA was separated on an oligo-dT cellulose column (type 3, Collaborative Biochemical Products). Ventricle poly(A) RNA was fractionated by centrifugation (18 h, 30,000 g, 4°C) on a linear 5%-25% sucrose gradient.

5 *Xenopus laevis* oocytes were prepared as previously described (Dascal & Lotan (1992) in *Methods in Molecular Biology*, v. 13: *Protocols in Molecular Neurobiology*, eds. Longstaff & Revest). and injected with either 50-120 ng/oocyte poly(A) RNA, 120-200 ng/oocyte total RNA, or 35 ng/oocyte fractionated poly(A) RNA. In most cases, 5HT1A-R RNA (5-20 ng/oocyte) was
10 co-injected with atrial or ventricle RNA. Final volume of the injected RNA solution was 50 nl. The oocytes were incubated for 3-7 days in the NDE solution (ND96 (see below) containing 1.8 mM CaCl₂ and supplemented with 2.5 mM Na-pyruvate and 50 µg/ml gentamicin). Occasionally, either 2.5-5% heat-inactivated horse serum or 0.5 mM theophylline were added to the NDE
15 solution. Incubation of oocytes in pertussis toxin (PTX; List Biochemicals) was done in NDE solution without the addition of pyruvate, serum or theophylline. cDNAs of 5HT1A receptor (Karschin *et al.* (1991) P.N.A.S. **88**:5694-5698) and G_{i2a} (a gift from M. I. Simon, Caltech) in pBluescript were linearized, and RNA was synthesized *in vitro* as described (Dascal and Lotan, *supra*).

20 Electrophysiological recordings were performed using the two electrode voltage clamp method with the Dagan 8500 amplifier (Dagan Instruments, Minneapolis) as described (Dascal *et al.* (1986) Mol. Brain Res. **1**:201-209). The oocytes were usually kept in the ND96 solution: 96 mM NaCl/2 mM KCl/1 mM MgCl₂/1 mM CaCl₂/5 mM Hepes, pH=7.5. Most measurements were done
25 in the high K⁺ solution (hK): 96 mM KCl/2 mM NaCl/1 mM MgCl₂/1 mM CaCl₂/5 mM Hepes, pH=7.5. Solutions containing intermediate concentrations of K⁺ were made by substituting K⁺ for Na⁺. Solution exchange and drug application were done by superfusing the cell placed in a 0.5 ml chamber. GDP-β-S

(trilithium salt; Sigma) was injected by pressure (Dascal *et al. supra.*). Stimulation, data acquisition, and analysis were performed using pCLAMP software (Axon Instruments, Foster City, CA).

5

RESULTS

To express the KG channel, the oocytes were injected with atrial total or poly(A) RNA. In order to avoid the possibility that a low level of expression of the muscarinic receptor will make undetectable even a well-expressed KG channel, atrial RNA was usually supplemented with mRNA coding for the serotonin-5HT1A receptor (5HT1A-R); oocytes injected with this RNA mixture will be termed RNA-injected oocytes throughout the paper. When expressed in atrial myocytes, the 5HT1A-R efficiently coupled to the KG channel normally existing in these cells (Karschin *et al. supra.*), and it was expected to do so in the oocytes.

Four to 5 days after RNA injection addition of 10 μ M ACh or 1-2 μ M 5HT to the ND96 bath solution did not cause any significant change in membrane current. Therefore, the effects of ACh and 5HT were tested in a high potassium (hK) solution with 96 mM K⁺ and 2 mM Na⁺. In this solution, the K⁺ equilibrium potential (E_K) is close to 0 mV, and this enables inward K⁺ current flow through inwardly rectifying K channels at negative holding potentials (-80 mV was routinely used in this study).

Changing ND 96 to the hK solution was accompanied by the development of an inward current that reached a steady level within 0.5-1 min (I_{hK} ; Fig 1A). I_{hK} was also observed in native (not injected with any RNA) oocytes, or in oocytes injected with 5HT1A-R RNA alone, but it was always larger in RNA-injected oocytes ($P < 0.001$, two-tailed t-test; Table 1).

Table 1

Inward currents evoked by high K^+ and by 5HT. The entries are inward currents in nA shown as mean \pm SEM (n), measured at -80mV in the hK solution. 5HT concentration ranged in different experiments from 100 nM to 2 μ M.

Injected RNA	I_{hK}	I_{5HT}
None (native oocytes)	72 \pm 6 (34)	0 (18)
5HT1A-R	54 \pm 4 (11)	0 (12)
Atrial + 5HT1A-R	123 \pm 8 (55)	290 \pm 43 (55)

5 In RNA-injected oocytes, application of 5HT or ACh in hK solution induced an inward current (I_{5HT}) that subsided upon washout of the transmitter (Fig. 1A, B). The response to ACh was usually smaller than to 5HT when measured in the oocytes of the same frog (Fig. 1B). Thus, in oocytes of one frog I_{5HT} was 1102 \pm 84 nA (n=6), whereas the ACh response was 382 \pm 45 nA(n=6). I_{5HT} tended to decrease on repeated applications of 5HT, and this could be overcome by increasing the intervals between applications to 10 min or more, suggesting the presence of a desensitization process. I_{5HT} and an increased (in comparison with native oocytes) I_{hK} were also observed in oocytes injected with ventricle poly (A) RNA + 5HT1A-R RNA, but the I_{5HT} was about 20 times smaller than with atrial poly(A) RNA (not shown). 5HT had no effect in oocytes injected with atrial RNA without the 5HT1A-R RNA (n=4) or with 5HT1A-R RNA alone, or in native oocytes (Table 1).

The 5HT dose-response curve showed saturation at about 100 nM and a half-maximal response at about 15 nM (Fig. 1C), which is characteristic of the 5HT1 receptor class (Hoyer & Schoeffer (1991) J. Recept. Res. 11:197-214). A similar current was evoked by a selective 5HT1A agonist, 8-OH DPAT (8-OH-2(D1-n-(propylamino)-tetralin).

The current-voltage (I-V) characteristic of the oocyte membrane was studied by applying voltage steps from a holding potential of -80 mV. In normal

ND96, in the range -140 - -20 mV, only voltage- and time-independent "leak" currents were observed (Fig. 2a), and the I-V curve was linear (Fig. 2B). Above -20 mV, a slowly developing outward current was observed (Fig. 2A, a-c); this is known to be due to opening of a Cl^- channel activated by Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Barish (1983) J. Physiol. **342**:309-325). The Ca^{2+} -activated Cl^- current was also seen in the hK solution; in addition, the total membrane current evoked by steps to -120 and up to -20 mV was larger than in ND96 (Fig. 2Ab; 2B), whereas above 0 mV there was little or no change. This suggested that most or all of I_{hK} elicited at -80 mV by the exchange of ND96 to hK solution was due to a K^+ current flowing through a constitutively active inward rectifier K^+ channel(s). This current showed some time-dependent inactivation at -140 mV (Fig. 2Ab) and at more negative potentials; this inactivation phenomenon was not studied further. In the presence of 5HT, the membrane currents between -140 and -20 mV were further increased (Fig. 2Ac). Net 5HT-evoked currents, obtained by digital subtraction of total membrane currents in the absence of 5HT from currents in its presence (Fig. 2Ad), showed clear inward rectification; the 5HT-activated channels conducted little or no current above E_{K} at different external K^+ concentrations, $[\text{K}_{\text{out}}]$ (Fig. 2C). The extrapolated reversal potential of $I_{5\text{HT}}$ showed an almost perfect selectivity of the 5HT-activated channel to K^+ , changing by about 58 mV per 10-fold change in $[\text{K}_{\text{out}}]$ (Fig. 2D). The reversal potential of the total membrane current in the absence of 5HT also depended on $[\text{K}_{\text{out}}]$ (Fig. 2B) but changed only by 24 mV per tenfold change in $[\text{K}_{\text{out}}]$ (Fig. 2D). This does not necessarily imply poor ion selectivity of the constitutively active inward rectifier, but may reflect the relatively high contribution of Cl^- and Na^+ to the resting membrane conductance (Dascal *et al.* (1984) J. Physiol. **352**:551-574).

Block by external Ba^{2+} is one of the characteristic features of inward rectifiers (Hille, *supra.*). In normal ND96 solution, Ba^{2+} (5 μ M-3 mM) did not cause any significant changes in resting current or conductance in native or RNA-injected oocytes at the holding potential of -80mV. In the hK solution, Ba^{2+} inhibited both I_{hK} and I_{5HT} (Fig. 3), and this was accompanied by a decrease in membrane conductance. 300 μ M, Ba^{2+} blocked about 20% of I_{hK} but almost completely abolished I_{5HT} (Fig. 3B). The IC_{50} (half-inhibition concentration) for Ba^{2+} block of I_{5HT} was about 15 μ M, whereas IC_{50} for I_{hK} block was above 3 mM (Fig. 3D). It is noteworthy that, although the sensitivity of I_{hK} to Ba^{2+} block was similar in native and RNA-injected oocytes, the latter did appear to have a small component of I_{hK} inhibited by low doses of Ba^{2+} (Fig. 3D). This raises the possibility that the atrial I_{hK} is more sensitive to Ba^{2+} block than the oocyte's I_{hK} , or that a fraction of the highly Ba^{2+} -sensitive channels underlying I_{5HT} could be active in the absence of agonist. Note also that there was an inward current "tail" observed when Ba^{2+} and 5HT was washed out simultaneously (Fig. 3B), presumably because the rate-limiting step in deactivation of the channel proceeds more slowly than unblock from Ba^{2+} .

To estimate the size of RNA encoding the expressed inward rectifiers, ventricle poly(A) RNA was fractionated on a sucrose gradient. The size distribution of the fractions was measured by RNA gel blots probed with [³²P]-labeled poly(T) (Lubbert *et al.* (1987) J. Neurosci. **7**:1159-1165). The RNA encoding I_{5HT} was found mainly in two size fractions covering the range between 2.5 and 5.5 kb. The peak expression of ventricle I_{hK} was in lower size fractions, in the 1.5-3 kb range.

In atrium, the muscarinic receptor is coupled to the KG channel via a PTX-sensitive G-protein. Surprisingly, in RNA-injected oocytes, I_{5HT} was not affected by treatment with PTX; neither was I_{hK} (Fig. 4A). To test whether the

5HT1A receptor couples to the K⁺ channel via a G-protein, the oocytes were injected with 400-800 pmole/oocyte of the non-hydrolysable analog of GDP, GDP-β-S, which is known to inhibit the activity of PTX-sensitive as well as of PTX-insensitive G-proteins (Gilman (1987) A. Rev. Biochem. 56:615-649). In 4 cells, GDP-β-S injection had no effect on I_{hK} (115±8% of control) but strongly inhibited I_{5HT}, to 4±1% of control (Fig. 4B). Thus, it appears that the coupling between the 5HT1A receptor and the KG channel occurs via an oocyte's endogenous PTX-insensitive G-protein.

We examined whether an overexpressed PTX-sensitive α subunit of a G-protein, e.g. G_{i2}α, could compete with the "native" PTX-insensitive α subunit for the expressed 5HT1A receptor, thus restoring the PTX sensitivity of the KG channel activation. As shown in Fig. 4A, in oocytes injected with atrial RNA plus cRNAs encoding 5HT1A-R and G_{i2}α, PTX inhibited I_{5HT} by about 50% (P<0.01, two-tailed t-test), whereas I_{hK} was unaffected.

DISCUSSION

The present results demonstrate for the first time that the atrial inward rectifier K⁺ (KG) channel, which in the native tissue is activated by ACh via a PTX-sensitive G-protein, is expressed in oocytes injected with atrial RNA. Current through the channel can be activated by acetylcholine (ACh) or, if RNA encoding a neuronal 5HT1A receptor is co-injected with atrial RNA, by serotonin (5HT). Activation of the channel probably occurs via a muscarinic ACh receptor synthesized following atrial RNA injection, rather than via the oocyte's endogenous muscarinic receptor. The latter couples to phospholipase C, and its activation induces very characteristic large transient Cl⁻ current responses caused by Ca²⁺ release from intracellular stores (Dascal (1987) CRC Crit. Rev. Biochem. 22:317-387). Fortunately, the majority of oocyte batches lose this response after defolliculation (Miledi & Woodward

(1989) J. Physiol. **416**:601-621), and this response was not observed in the present study. Because the ACh-evoked currents were small in most cases, we concentrated on the study of the 5HT response; the latter was undoubtedly mediated by the introduced 5HT1A receptor, as 5HT was ineffective in oocytes not injected with 5HT1A-RNA, and the response displayed the expected pharmacological properties.

The evidence presented here indicates that, in oocytes injected with atrial and 5HT1A-R RNAs, activation of the 5HT1A receptor leads to opening of a K⁺ channel that bears distinctive features of an inward rectifier, similar to those of the atrial KG: i) it conducts inward but not outward K⁺ current; ii) it is blocked by low concentrations of Ba²⁺, iii) the conductance of the channel does not depend solely on voltage but on (E-E_K). The expression of this channel must truly be directed by atrial RNA, because: i) no hormone or transmitter-activated current of this kind is observed in native oocytes; ii) expression of 5HT1A receptor alone does not cause the appearance of such a response. Based on ventricle RNA fractionation data, the RNA encoding the 5HT-activated channel is in a broad size range between 2.5 and 5.5 kb. This is similar or somewhat smaller than the reported 4-5 kb mRNA size of some constitutively active inward rectifiers expressed in *Xenopus* oocytes (Lewis *et al.* (1991) FEBS Lett. **290**:17-21; and Perier *et al.* (1992) J. Neurochem. **59**:1971-1974), as well as of the cloned IRK1 (5.5 kb) and ROMK1 (4 kb) channels. The properties of I_{5HT} directed by ventricle and atrial RNA are very similar, and it is reasonable to assume that they are encoded by the same RNA species.

Opening of the inward rectifier by 5HT is mediated by activation of a G-protein, as expected for the KG channel, because i) 5HT1A receptor belongs to the family of 7-helix receptors all of which act via G-proteins (Dascal (1987) *supra.*); ii) I_{5HT} was inhibited by intracellular injection of GDP-β-S. However, the G-protein participating in this pathway was PTX-insensitive, possibly an

endogenous oocyte G-protein. It is not clear why in the oocyte the channel activation pathway involves a PTX-insensitive G-protein. The atrial KG channel normally couples to G_i (Brown & Birnbaumer, *supra.*), and there are at least two subspecies of G_i in the oocyte (Olate *et al.* (1990) FEBS Lett. **268**:27-31); also, some G_i may be expressed from atrial RNA. Also, in the hippocampus, the 5HT1A receptor opens a K^+ channel by activating a PTX-sensitive G-protein. One possibility is that a vast excess of this undefined PTX-insensitive G-protein overrides the others in competition for coupling to the 5HT1A receptor. Whatever the reason for this unexpected coupling, our results show that the PTX sensitivity of the KG channel activation can be partially restored by overexpression of the α subunit of G_i . Since the actual identify of the α subunit does not seem to be important for activation of the expressed KG channel, these results imply that the $\beta\gamma$ subunit complex doublet may be the activator of the channel in this case.

Atrial and ventricle RNAs also induce an enhanced activity of an additional inward rectifier, that is active in the absence of any specific stimulation (referred to as I_{hK} herein). I_{hK} in atrial RNA-injected oocytes is about twice as large as in native oocytes or oocytes injected with 5HT1A-R RNA alone. This current does not appear to represent the "basal" activity of the same channel activated by 5HT or ACh because it has a much lower sensitivity to Ba^{2+} block. Moreover, the fractionation data indicates that the RNA directing the expression of I_{hK} is smaller than that encoding the KG channel. However, it is not clear whether this atrial (or ventricle) RNA encodes the channel itself or a factor that enhances the expression or the activity of a native channel. Further studies, such as expression cloning, will help to identify the messages encoding the two inward rectifiers whose expression is reported here.

Example 2

Formation of Heteromultimeric Kir3.0 Channels

Materials and methods

Plasmids and DNAs. To isolate Kir3.2 and Kir3.3 from mouse brain RNA, oligonucleotides were designed that anneal to the first assigned methionine codon and the assigned stop codon of mouse Kir3.2 and Kir3.3 (Lesage *et al* 5 (1994) FEBS Lett. **353**:37-42). Kir3.0-specific sequences were coupled to a 5'-end untranslated sequence from alfalfa mosaic virus (Jobling and Gehrke (1987) Nature **325**:622-625) and a T7 RNA polymerase recognition site to confer an optimal translational initiation site and allow RNA synthesis *in vivo* 10 (cRNA). At the 3' end, a poly(A) tail was added to confer RNA stability. Total RNA from adult mouse brain (purchased from Clontech) was used as template for cDNA synthesis (Sambrook *et al.*, *supra.*). PCR was conducted in a thermal cycler (Perkin-Elmer) by using Vent Polymerase (New England Biolabs), which minimizes misincorporations during the amplification step. Kir3.2 and Kir3.3 15 PCR-derived sequences were then inserted into the pNoTA vector (5 Prime → 3 Primer, Inc.) for subsequent sequence analysis.

Kir3.1 cloned from cardiac atrium and inserted originally in pBluescript (Stratagene) was transferred to the vector pMXT (gift from J. Yang; University of Texas, Dallas), in which the cloning site is flanked by 5' and 3' untranslated 20 sequences from *Xenopus* globin. G protein subunits G β 1 and G γ 2 cDNAs were in the pFroggy vector (gift from L. Jan, University of California, San Francisco), as described in Lim *et al.* (1995) J. Gen. Physiol. **105**:421-439. The m2R cDNA (gift from E. Peralta, Harvard University) was in the pGEM3 vector (Promega).

RNA Synthesis and Oocyte Injections. Kir3.2 and Kir3.3 cRNAs were 25 synthesized directly from gel-isolated PCR products, while the remaining cRNAs were synthesized from linearized plasmid DNAs. cRNAs were dissolved in sterile water and injected into stage V or VI *Xenopus* oocytes as described in Quick and Lester (1994) in *Ion Channels of Excitable Cells*, ed.

Narahashi (Academic, San Diego) pp.261-279. Oocytes were maintained in ND96 solution. Oocytes were assayed 2-5 days after injection.

Electrophysiology. Whole-cell currents from oocytes were measured by using an Axoclamp 2A or Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) in the two-electrode, voltage-clamp configuration. Current and voltage electrodes were filled with 3 M KCl to yield resistances ranging from 0.5 to 1.5 MΩ. Recordings were started in an external solution (0 K⁺) containing 98 mM NaCl, 1 mM MgCl₂, and 5 mM Hepes, pH 7.3. In high-K⁺ -containing solutions, the NaCl was replaced either completely by 98 mM KCl (98 K⁺), or partially, by 20 mM KCl (20 K⁺).

Cell-attached recordings of single channels were recorded from *Xenopus* oocytes as described in Methfessel et al. (1986) Pflugers Arch. 407, 577-588. Pipette solutions contained 150 mM KCl, 1 mM CaCl₂, and 5 mM Hepes, pH 7.3 with KOH. Bath solution contained 150 mM KCl, 1 mM MgCl₂, 1 mM EGTA, and 5 mM Hepes, pH 7.3 with KOH. For single-channel analysis, the current traces were filtered at 2 kHz and sampled at 10 kHz. Current amplitude histograms and open-time durations were obtained by using FETCHAN and pSTAT from pCLAMP 6.0 (Axon Instruments). All recordings were performed at room temperature (≈22°C).

RESULTS

Kir3.1 is abundantly expressed in cardiac atrium and brain. When heterologously expressed in *Xenopus* oocytes, Kir3.1 induces strong inwardly rectifying K⁺ currents either with m2R activation or with coexpression of G-protein subunits Gβ1 and Gγ2.

Because expression of Kir3.2 and Kir3.1 was consistently smaller than for other channels expressed in oocytes, the effects of Kir3.1 and Kir3.2 coexpression under conditions of maximal m2R activation [1 μM acetylcholine

(ACh)] were examined. With the coinjection of Kir3.1 and Kir3.2 (Kir3.1 + 2) cRNAs (0.5 ng each per oocyte), exposure to ACh in these oocytes led to development of large inward currents when the voltage was jumped from 0 mV to more negative values. By contrast, in oocytes injected with Kir3.2 or Kir3.1 cRNAs (1 ng), receptor activated currents were much smaller in amplitude, comparable to levels reported previously. On average, Kir3.1 + 2 currents [inward currents induced by ACh ($I_{K,ACh}$) = $-6.4 \pm 0.8 \mu A$; mean \pm SEM; $n = 5$] were about 9-fold larger than Kir3.2 currents ($I_{K,ACh}$ = -698 ± 98 nA; $n = 7$) and 17-fold larger than Kir3.1 currents ($I_{K,ACh}$ = -365 ± 45 nA; $n=5$).

Kir3.1 expressed in oocytes shows distinctive gating kinetics, including slow phases of activation (several hundred ms) during a jump from 0 mV to more negative potentials. For a similar voltage jump, Kir3.2 expressed in oocytes showed kinetics that more closely resemble other strong inward rectifiers, with a prominent phase of inactivation. These differences may be governed at least partially by differences in the sequence of the P region. The coexpressed subunits showed a slow phase of activation, but the time course of this activation cannot be explained as a simple weighted sum of the waveforms for Kir3.1 alone and Kir3.2 alone. Thus, the relations for jumps to voltages between -60 and -140 mV were well described by two exponential components with nearly voltage-independent time constants; at -80 mV the time constant of the slower component was 213 ± 12 ms ($n = 4$), or less than half that for Kir3.1 (32). These kinetic differences indicate molecular interactions between the Kir3.1 and Kir3.2 channels.

The large enhancement of the agonist-evoked currents for coexpressed channels might be explained by effects on any component in the receptor-channel signaling pathway, including receptors, endogenous G proteins, or the channels themselves. To discriminate among these possibilities, the receptor was uncoupled from the channel by overexpression of G protein subunits $G\beta 1$

and G γ 2. Importantly, cells expressing G β 1 γ 2 and Kir3.2 showed persistent inwardly rectifying currents at amplitudes comparable or larger to those observed for m2R activation of Kir3.2. Therefore, Kir3.2, like Kir3.1, is activated by G β γ .

5 Cells coexpressing Kir3.2 and Kir3.1 (Kir3.1 + 2) responded to high-K⁺ solution with much larger currents than did cells expressing only Kir3.1 or Kir3.2, consistent with the results obtained with m2R activation. On average, Kir3.1 + 2 currents ($I_K = -4.3 \pm 1 \mu A$; $n = 7$) were 14 times larger than Kir3.2 currents ($I_K = -305 \pm 56 \text{ nA}$; $n = 7$) and 40 times larger than Kir3.1 currents ($I_K =$
10 $-104 \pm 10 \text{ nA}$; $n = 6$). Thus, these experiments demonstrate that the large mutual potentiating effect of Kir3.1 + 2 is independent of the method of G-protein activation and argue that the effects are on the channels themselves.

 An increase in the number of channels or modification of the intrinsic channel properties might account for this potentiation. Single-channel
15 recordings of Kir3.1 + 2 in combination or of Kir3.2 alone were made to test these possibilities. In oocytes coinjected with cRNAs for Kir3.2, G β 1, and G γ 2, single-channel currents in the cell-attached configuration displayed features consistent with macroscopic measurements, *i.e.* with hyperpolarization of the unit conductance increased. No outward currents were detected at membrane
20 potentials positive to the K⁺ equilibrium potential ($E_K; \approx 0 \text{ mV}$). These channels openings had a mean slope conductance of $30 \pm 2 \text{ pS}$ ($n = 4$) over the range from -40 to -100 mV , significantly smaller than the value of 39 pS for Kir3.1 alone, and showed bursts of flicker activity. Mean open-time distribution could be described by a fast component of 0.1 ms (35% of the total number of events)
25 and a main slower component of 0.5 ms (65% of the total number of events).

 The combination of Kir3.1 + 2 produced unitary currents with strong inward rectification and a mean slope conductance of $35 \pm 3 \text{ pS}$ ($n = 4$), intermediate between the values for Kir3.1 alone and Kir3.2 alone. In addition,

Kir3.1 + 2 channels displayed markedly longer openings than Kir3.2 channels. Recordings from Kir3.1 + 2 channels showed a 7-fold increase in the major component of open-time duration (3.5 ms; 71% of the total number of events; there was also a smaller component of 0.5 ms; 29% of the total number of events) compared to Kir3.2. Qualitatively, the Kir3.1 + 2 patch recordings are rather similar to those observed for Kir3.1 in terms of mean open time, although the typically low expression levels for Kir3.1 alone vitiate systematic comparisons. At present, it is not known whether this increase, like the increase in macroscopic currents on Kir3.1 + 2 coexpression, is solely explained by the increase in open-time duration or whether the enhanced currents also arise from an increase in the number of openings, either because each channel opens more often or because there are more functional channels.

The effects of Kir3.3 coexpression was tested with Kir3.1 or Kir3.2. Injection of Kir3.3, G β ₁, and G γ ₂ cRNAs did not activate inward currents in high-K⁺-containing solution; nor were currents activated by opioid receptors. Nevertheless, Kir3.3 had profound effects when coexpressed with Kir3.1. Coexpression of Kir3.1 and Kir3.3 (Kir3.1 + 3) resulted in 7-fold larger I_K currents (I_K = -3.4 ± 0.5 μA; n = 6) than for Kir3.1 alone (I_K = -481 ± 43 nA; n = 6). Such augmentation was seen for each of several batches of oocytes and cRNAs and on activation of the m2R pathway. Thus, these results demonstrate that Kir3.3, despite not being directly activated by G protein subunits G β ₁ and G γ ₂, can interact with, and increase, G-protein-mediated responses of Kir3.1.

Surprisingly, Kir3.3 had a suppressing effect when coexpressed with Kir3.2. G β γ -induced, inwardly rectifying currents in oocytes coinjected with Kir3.2 and Kir3.3 cRNAs (0.5 ng each per oocyte) (I_K = -129 ± 22 nA; n = 7) were much smaller than in oocytes injected with Kir3.2 cRNA alone (1 ng per

oocyte) $I_K = -5.1 \pm 0.6 \mu A$; $n = 9$). This dominant-negative effect of GIRk3 on Kir3.2 currents was also observed when m2R was coexpressed, instead of G-protein subunits $G\beta_1$ and $G\gamma_2$.

A very important issue is raised by the occurrence of heteromultimeric channels is their differential distribution in excitable tissues and their physiological significance. Distinct Kir3.0 heteromultimeric channels may be modulated or activated by G proteins differentially, as reported for other G protein effectors. This allows for a larger degree of flexibility in the control of this important form of neuronal signaling.

The plasmid pBSIIKS(-)KGA, encoding the rat inward rectifier, G-protein activated, mammalian, potassium KGA channel was deposited on May 17, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., under the provisions of the Budapest Treaty for the International Recognition of the Deposition of Microorganism for the Purposes of Patent Procedure, and given the ATCC accession number 75469.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated DNA molecule encoding at least two different inward rectifier, G-protein activated, mammalian, potassium Kir3.0 polypeptides, wherein said nucleic acid is characterized by its ability to cause a change in potassium flow across a *Xenopus* oocyte cell membrane upon expression therein.
2. A nucleic acid according to Claim 1, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.2.
3. A nucleic acid according to Claim 1, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.3.
4. A nucleic acid according to Claim 1, wherein said mammalian Kir3.0 polypeptides are Kir3.2 and Kir3.3.
5. A nucleic acid according to Claim 2, wherein said Kir3.1/KGA polypeptide has the amino acid sequence of SEQ ID NO:2.
6. A method of producing a functional Kir3.0 channel in an expression host cell, the method comprising:
 - introducing into said expression host cell a nucleic acid encoding a first mammalian Kir3.0 polypeptide and a nucleic acid encoding a second mammalian Kir3.0 polypeptide into said heterologous cell under conditions that permit expression of said nucleic acids;
 - wherein said mammalian Kir3.0 polypeptides assemble to form a functional Kir3.0 in said expression host cell.

7. A method according to Claim 6, wherein said nucleic acid encoding said first mammalian Kir3.0 polypeptide and said nucleic acid encoding said second mammalian Kir3.0 polypeptide are present on a single vector.

5

8. A method according to Claim 6, wherein said nucleic acid encoding said first mammalian Kir3.0 polypeptide and said nucleic acid encoding said second mammalian Kir3.0 polypeptide are present on different vectors.

10

9. A method according to Claim 6, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.2.

10. A method according to Claim 6, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.3.

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11. A method according to Claim 6, wherein said mammalian Kir3.0 polypeptides are Kir3.2 and Kir3.3.

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12. A host cell comprising a functional heterologous Kir3.0 channel; wherein said functional Kir3.0 channel comprises a first Kir3.0 polypeptide and a second mammalian Kir3.0 polypeptide assembled to form a functional Kir3.0 channel.

25

13. A host cell according to Claim 12, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.2.

14. A host cell according to Claim 12, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.3.

15. A host cell according to Claim 12, wherein said mammalian
5 Kir3.0 polypeptides are Kir3.2 and Kir3.3.

16. A host cell according to Claim 12, wherein said host cell is a *Xenopus laevis* oocyte.

10 17. A method of screening for agents that modulate the activity of a Kir3.0 channel, the method comprising:
combining a candidate agent with a functional Kir3.0 channel under conditions that permit inward K⁺ current
determining the induced current;
15 wherein a change in said induced current in the presence of said agent as compared to a control is indicative that said agent modulates the activity of a Kir3.0 channel.

Patented 2006-06-06

INWARD RECTIFIER, G-PROTEIN ACTIVATED, MAMMALIAN, POTASSIUM
CHANNELS AND USES THEREOF

5

ABSTRACT OF THE DISCLOSURE

Compositions and methods are provided for producing functional mammalian inward rectifier, G-protein activated potassium channels (Kir3.0 channels). A channel is a multimeric protein comprising one or more Kir3.0 polypeptides, e.g. Kir3.1, Kir3.2, etc., where the polypeptides may be from the same or different species. The functional channel has the distinctive features of an anomalous rectifier, in that it conducts inward but not outward K^+ current; it is blocked by low concentrations of extracellular Cs^+ or Ba^{2+} ; and the conductance of the channel does not depend solely on voltage, but on $(E-E_K)$. The ability of the channel to conduct inward K^+ current is modulated by G-proteins, particularly G-proteins of the G_i/G_o family. A number of mammalian cell surface receptors activate G-proteins as a consequence of specific ligand binding. The signal transduction from receptor to Kir3.0 channel is therefore coupled through G-protein intermediates.

The functional Kir3.0 channels are useful in drug screening assays directed to modulation of cellular electrophysiology. Nucleic acids encoding Kir3.0 polypeptides are useful for expression of the gene product, and for identification of homologous genes from other species, as well as other members of the same family of proteins. Expression of the nucleic acids in a heterologous cell, e.g. *Xenopus* oocyte, confers the ability to cause a change in potassium flow in response to G-protein activation.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 2

CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: FLEHR, HOHBACH, TEST, ALBRITTON & HERBERT
(B) STREET: 4 Embarcadero Center, Suite 3400
(C) CITY: San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94111-4187

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(A) NAME: Trecartin, Richard F.
(B) REGISTRATION NUMBER: 31,801
(C) REFERENCE/DOCKET NUMBER: A63098/RFT

(A) TELEPHONE: (415) 781-1989
(B) TELEFAX: (415) 398-3249
(C) TELEX: 910 277299

(i) SEQUENCE CHARACTERISTICS:

SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2076 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 32..1534

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCACGAGAA TCTGGATCTC CCCTCCGTAT T ATG TCT GCA CTC CGA AGG AAA 52
Met Ser Ala Leu Arg Arg Lys
1 5

TTT GGG GAC GAT TAC CAG GTA GTG ACC ACT TCG TCC AGC GGT TCG GGC 100
Phe Gly Asp Tyr Gln Val Thr Thr Ser Ser Ser Gly Ser Gly
10 15 20

TTG CAG CCC CAG GGG CCA GGA CAG GGC CCA CAG CAG CAG CTT GTA CCC 148
Leu Gln Pro Gln Gly Pro Gly Gln Gly Pro Gln Gln Gln Leu Val Pro
25 30 35

AAG Lys 40	AAG Lys	AAA Lys	CGG Arg	CAG Gln	CGG Arg 45	TTC Phe	GTG Val	GAC Asp	AAG Lys	AAC Asn 50	GGT Gly	CGG Arg	TGC Cys	AAT Asn	GTG Val 55	196
CAG Gln	CAC His	GGC Gly	AAC Asn	CTG Leu 60	GGC Gly	AGC Ser	GAG Glu	ACC Thr	AGT Ser 65	CGC Arg	TAC Tyr	CTT Leu	TCC Ser	GAC Asp 70	CTC Leu	244
TTC Phe	ACT Thr	ACC Thr	CTG Leu 75	GTG Val	GAT Asp	CTC Leu	AAG Lys	TGG Trp 80	CGT Arg	TGG Trp	AAC Asn	CTC Leu	TTT Phe 85	ATC Ile	TTC Phe	292
ATC Ile	CTC Leu	ACC Thr 90	TAC Tyr	ACC Thr	GTG Val	GCC Ala	TGG Trp 95	CTC Leu	TTC Phe	ATG Met	GCG Ala	TCC Ser 100	ATG Met	TGG Trp	TGG Trp	340
GTG Val 105	ATC Ile	GCT Ala	TAT Tyr	ACC Thr	CGG Arg	GGC Gly 110	GAC Asp	CTG Leu	AAC Asn	AAA Lys	GCC Ala 115	CAT His	GTC Val	GGC Gly	AAC Asn	388
TAC Tyr 120	ACT Thr	CCC Pro	TGT Cys	GTG Val	GCC Ala 125	AAT Asn	GTC Val	TAT Tyr	AAC Asn	TTC Phe 130	CCC Pro	TCT Ser	GCC Ala	TTC Phe	CTT Leu 135	436
TTC Phe	TTC Phe	ATC Ile	GAG Glu	ACC Thr 140	GAG Glu	GCC Ala	ACC Thr	ATC Ile	GGC Gly 145	TAT Tyr	GGC Gly	TAC Tyr	CGC Arg	TAC Tyr 150	ATC Ile	484
ACC Thr	GAC Asp	AAG Lys 155	TGC Cys	CCC Pro	GAG Glu	GGC Gly	ATC Ile	ATC Ile 160	CTT Leu	TTC Phe	CTT Leu	TTC Phe	CAG Gln 165	TCC Ser	ATC Ile	532
CTT Leu	GGC Gly	TCC Ser 170	ATC Ile	GTG Val	GAC Asp	GCT Ala	TTC Phe	CTC Leu	ATC Ile	GGC Gly	TGC Cys	ATG Met 180	TTC Phe	ATC Ile	AAG Lys	580
ATG Met 185	TCC Ser	CAG Gln	CCC Pro	AAA Lys	AAG Lys	CGC Arg 190	GCC Ala	GAG Glu	ACC Thr	CTC Leu	ATG Met 195	TTT Phe	AGC Ser	GAG Glu	CAT His	628
GCG Ala 200	GTT Val	ATT Ile	TCC Ser	ATG Met	AGG Arg 205	GAC Asp	GGA Gly	AAA Lys	CTC Leu	ACT Thr 210	CTC Leu	ATG Met	TTC Phe	CGG Arg	GTG Val 215	676
GGC Gly	AAC Asn	CTG Leu	CGC Arg	AAC Asn 220	AGC Ser	CAC His	ATG Met	GTC Val	TCC Ser 225	GCG Ala	CAG Gln	ATC Ile	CGC Arg	TGC Cys 230	AAG Lys	724
CTG Leu	CTC Leu	AAA Lys	TCT Ser 235	CGG Arg	CAG Gln	ACA Thr	CCT Pro	GAG Glu 240	GGT Gly	GAG Glu	TTT Phe	CTA Leu	CCC Pro 245	CTT Leu	GAC Asp	772
CAA Gln 250	CTT Leu	GAA Glu 250	CTG Leu	GAT Asp	GTA Val	GGT Gly	TTT Phe 255	AGT Ser	ACA Thr	GGG Gly	GCA Ala 260	GAT Asp 260	CAA Gln	CTT Leu	TTT Phe	820
CTT Leu 265	GTG Val	TCC Ser	CCT Pro	CTC Leu	ACC Thr	ATT Ile 270	TGC Cys	CAC His	GTG Val	ATC Ile	GAT Asp 275	GCC Ala	AAA Lys	AGC Ser	CCC Pro	868
TTT Phe 280	TAT Tyr	GAC Asp	CTA Leu	TCC Ser	CAG Gln 285	CGA Arg	AGC Ser	ATG Met	CAA Gln	ACT Thr 290	GAA Glu	CAG Gln	TTC Phe	GAG Glu	GTG Val 295	916
GTC Val	GTC Val	ATC Ile	CTG Leu	GAA Glu 300	GGC Gly	ATC Ile	GTG Val	GAA Glu	ACC Thr 305	ACA Thr	GGG Gly	ATG Met	ACT Thr	TGT Cys 310	CAA Gln	964

[illegible]

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 501 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Ala Leu Arg Arg Lys Phe Gly Asp Asp Tyr Gln Val Val Thr
1 5 10 15
Thr Ser Ser Ser Gly Ser Gly Leu Gln Pro Gln Gly Pro Gly Gln Gly
20 25 30
Pro Gln Gln Gln Leu Val Pro Lys Lys Lys Arg Gln Arg Phe Val Asp
35 40 45
Lys Asn Gly Arg Cys Asn Val Gln His Gly Asn Leu Gly Ser Glu Thr
50 55 60
Ser Arg Tyr Leu Ser Asp Leu Phe Thr Thr Leu Val Asp Leu Lys Trp
65 70 75 80
Arg Trp Asn Leu Phe Ile Phe Ile Leu Thr Tyr Thr Val Ala Trp Leu
85 90 95
Phe Met Ala Ser Met Trp Trp Val Ile Ala Tyr Thr Arg Gly Asp Leu
100 105 110
Asn Lys Ala His Val Gly Asn Tyr Thr Pro Cys Val Ala Asn Val Tyr
115 120 125
Asn Phe Pro Ser Ala Phe Leu Phe Phe Ile Glu Thr Glu Ala Thr Ile
130 135 140
Gly Tyr Gly Tyr Arg Tyr Ile Thr Asp Lys Cys Pro Glu Gly Ile Ile
145 150 155 160
Leu Phe Leu Phe Gln Ser Ile Leu Gly Ser Ile Val Asp Ala Phe Leu
165 170 175
Ile Gly Cys Met Phe Ile Lys Met Ser Gln Pro Lys Lys Arg Ala Glu
180 185 190
Thr Leu Met Phe Ser Glu His Ala Val Ile Ser Met Arg Asp Gly Lys
195 200 205
Leu Thr Leu Met Phe Arg Val Gly Asn Leu Arg Asn Ser His Met Val
210 215 220
Ser Ala Gln Ile Arg Cys Lys Leu Leu Lys Ser Arg Gln Thr Pro Glu
225 230 235 240
Gly Glu Phe Leu Pro Leu Asp Gln Leu Glu Leu Asp Val Gly Phe Ser
245 250 255
Thr Gly Ala Asp Gln Leu Phe Leu Val Ser Pro Leu Thr Ile Cys His
260 265 270
Val Ile Asp Ala Lys Ser Pro Phe Tyr Asp Leu Ser Gln Arg Ser Met
275 280 285
Gln Thr Glu Gln Phe Glu Val Val Val Ile Leu Glu Gly Ile Val Glu
290 295 300
Thr Thr Gly Met Thr Cys Gln Ala Arg Thr Ser Tyr Thr Glu Asp Glu
305 310 315 320

090999Z "0346600000

FIGURE 1A

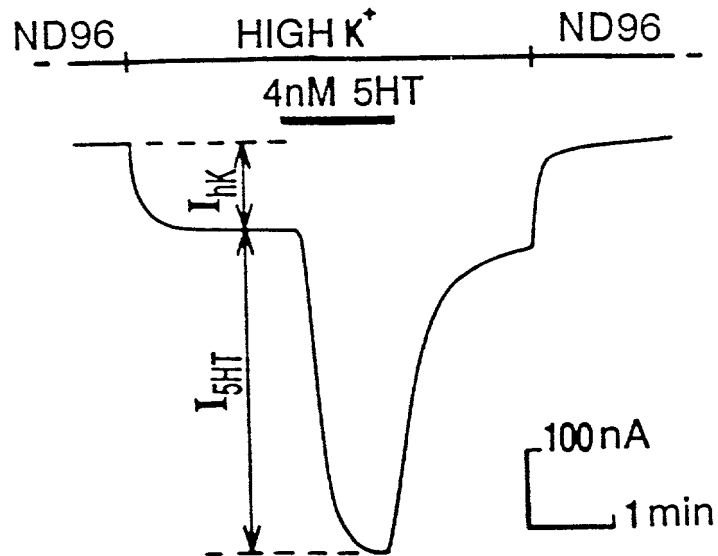


FIGURE 1B

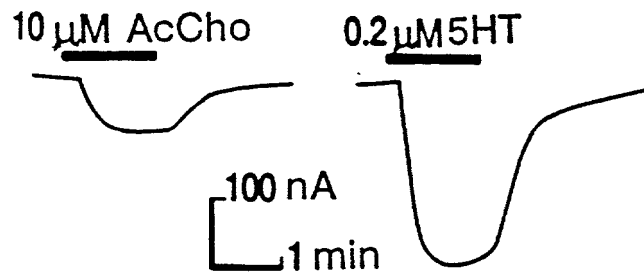


FIGURE 1C

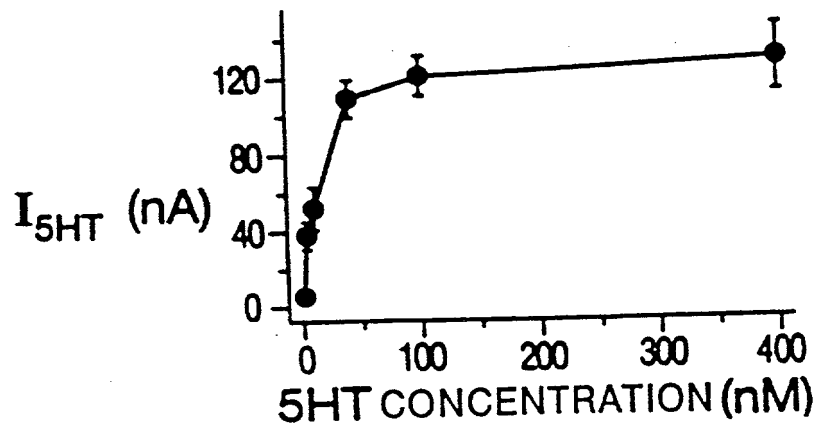


FIGURE 2A

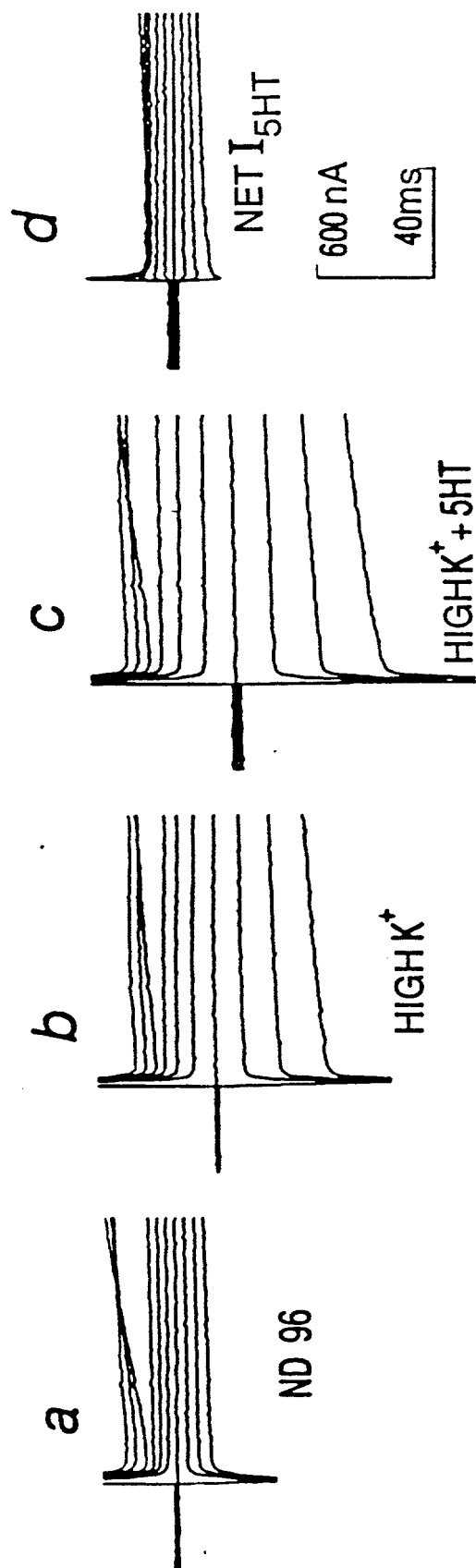


FIGURE 2B

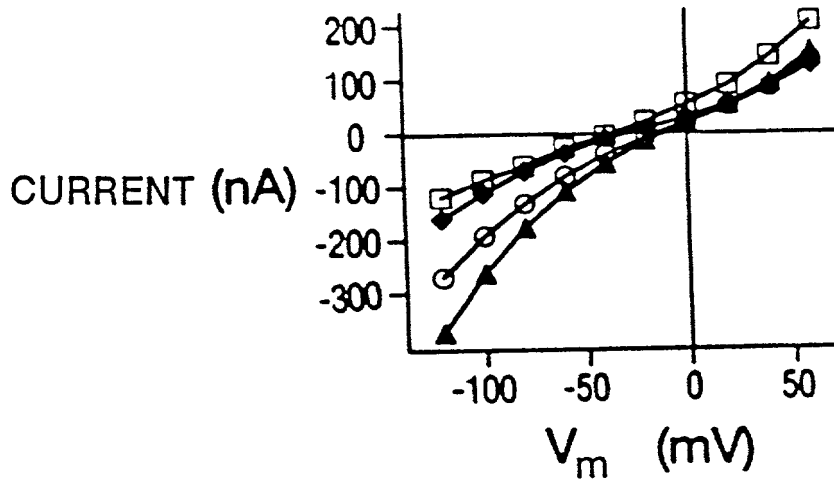


FIGURE 2C

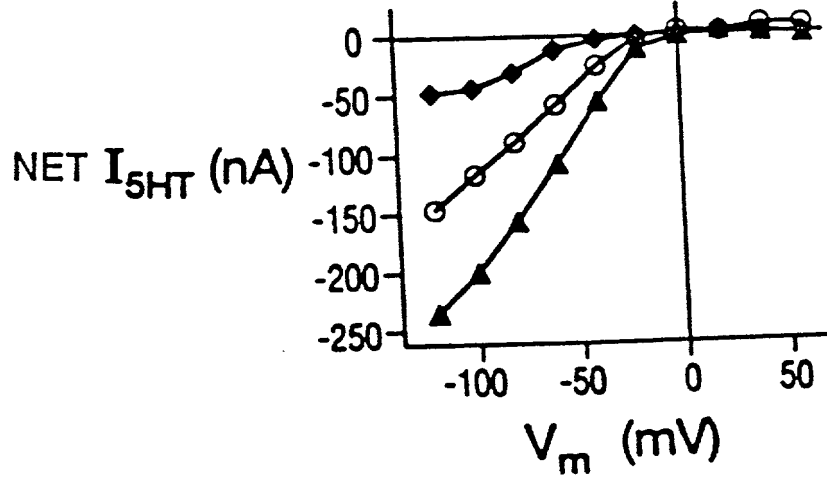


FIGURE 2D

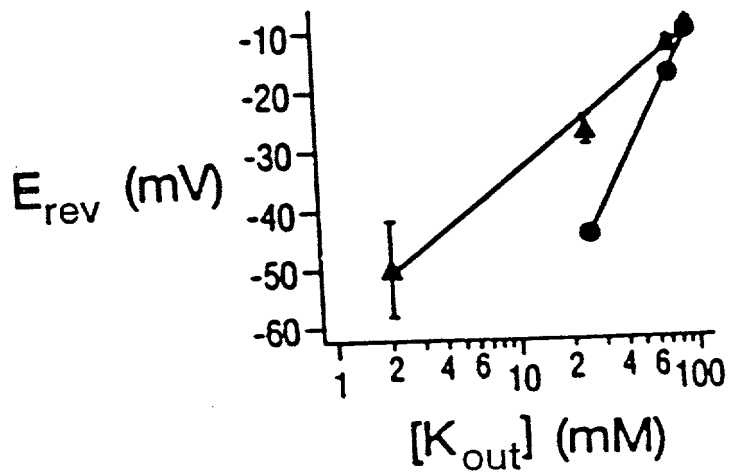


FIGURE 3A

FIGURE 3B

FIGURE 3C

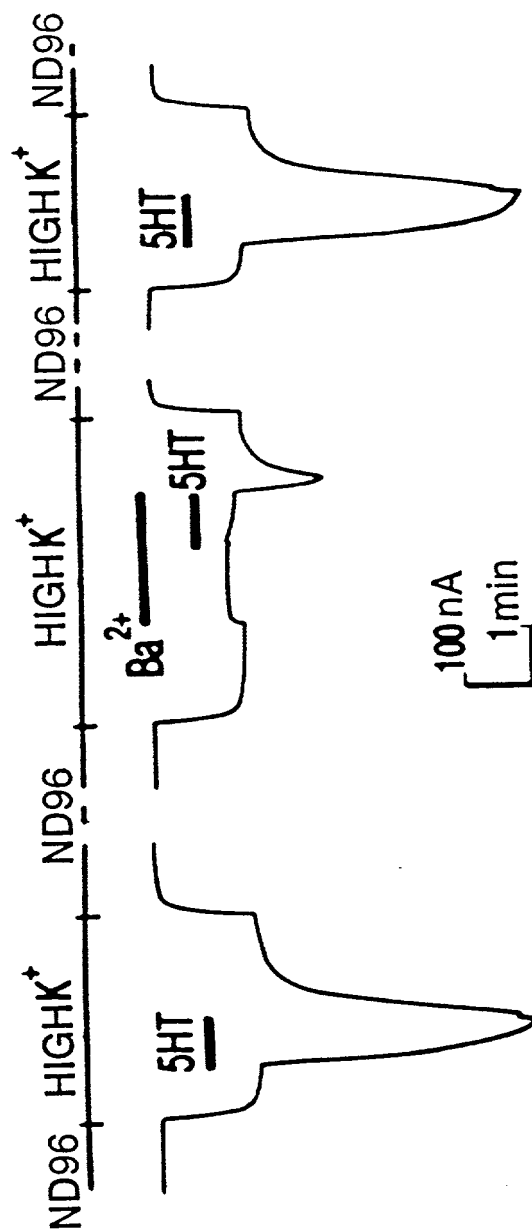


FIGURE 3D

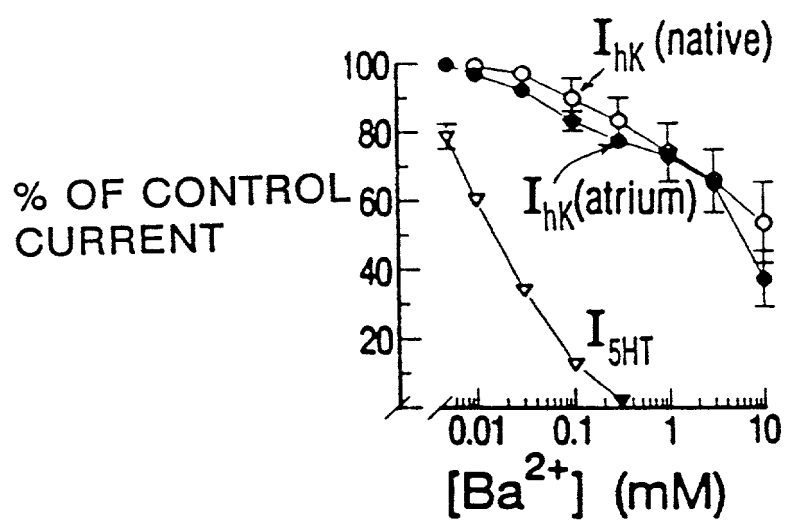


FIGURE 4A

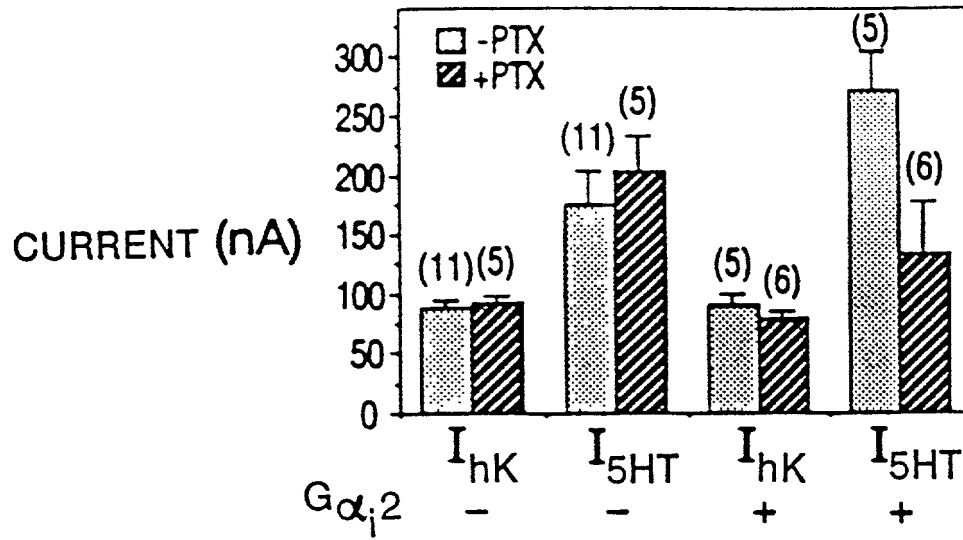
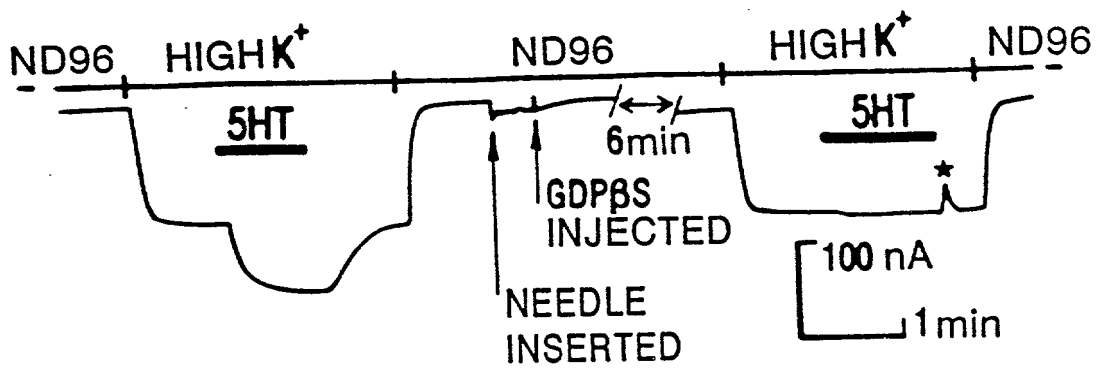


FIGURE 4B



DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled INWARD RECTIFIER, G-PROTEIN ACTIVATED, MAMMALIAN, POTASSIUM CHANNELS AND USES THEREOF, the specification of which

(check
one)

☐

is attached hereto.

☒

was filed on 7 March 1996 as

Application Serial No. _____

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

Priority Claimed

(Number) (Country) (Day/Month/Year Filed)

☐
☐

Yes

No

(Number) (Country) (Day/Month/Year Filed)

☐
☐

Yes

No

(Number) (Country) (Day/Month/Year Filed)

☐
☐

Yes

No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

08/066,371
(Application Serial No.)

21 March 1993
(Filing Date)

Pending
(Status)
(patented, pending, abandoned)

Variable	Mean	SD	Min	Max
1. Age	35.2	12.5	18	65
2. Sex	0.45	0.50	0	1
3. Education	12.8	2.1	8	16
4. Income	25.3	15.7	10	50
5. Health	4.2	1.8	1	7
6. Stress	3.8	1.5	1	6
7. Sleep	5.1	2.0	2	8
8. Diet	4.5	1.9	2	7
9. Exercise	3.2	1.6	1	5
10. Social Support	4.8	2.2	2	7
11. Coping Strategies	3.5	1.7	1	6
12. Resilience	4.1	1.9	2	7
13. Life Satisfaction	3.9	1.6	1	6
14. Mental Health	4.3	1.8	2	7
15. Physical Health	4.6	1.9	2	7
16. Work-Life Balance	3.7	1.7	1	6
17. Financial Stability	3.4	1.6	1	6
18. Family Harmony	4.0	1.8	2	7
19. Personal Growth	3.6	1.7	1	6
20. Community Involvement	3.3	1.5	1	5
21. Self-Esteem	4.4	1.9	2	7
22. Optimism	4.2	1.8	2	7
23. Gratitude	4.1	1.7	2	7
24. Mindfulness	3.8	1.6	1	6
25. Emotional Regulation	3.9	1.7	1	6
26. Stress Management	3.7	1.6	1	6
27. Resilience Training	3.5	1.5	1	5
28. Life Skills	3.6	1.6	1	6
29. Problem Solving	3.4	1.5	1	5
30. Decision Making	3.3	1.4	1	5
31. Time Management	3.2	1.3	1	5
32. Organization	3.1	1.2	1	4
33. Planning	3.0	1.1	1	4
34. Prioritization	2.9	1.0	1	4
35. Delegation	2.8	0.9	1	4
36. Collaboration	2.7	0.8	1	4
37. Communication	2.6	0.7	1	4
38. Teamwork	2.5	0.6	1	4
39. Leadership	2.4	0.5	1	4
40. Innovation	2.3	0.4	1	4
41. Creativity	2.2	0.3	1	4
42. Risk Taking	2.1	0.2	1	4
43. Adaptability	2.0	0.1	1	4
44. Flexibility	1.9	0.1	1	4
45. Openness	1.8	0.1	1	4
46. Conscientiousness	1.7	0.1	1	4
47. Neuroticism	1.6	0.1	1	4
48. Extraversion	1.5	0.1	1	4
49. Agreeableness	1.4	0.1	1	4
50. Openness to Experience	1.3	0.1	1	4
51. Conscientiousness	1.2	0.1	1	4
52. Neuroticism	1.1	0.1	1	4
53. Extraversion	1.0	0.1	1	4
54. Agreeableness	0.9	0.1	1	4
55. Openness to Experience	0.8	0.1	1	4
56. Conscientiousness	0.7	0.1	1	4
57. Neuroticism	0.6	0.1	1	4
58. Extraversion	0.5	0.1	1	4
59. Agreeableness	0.4	0.1	1	4
60. Openness to Experience	0.3	0.1	1	4
61. Conscientiousness	0.2	0.1	1	4
62. Neuroticism	0.1	0.1	1	4
63. Extraversion	0.0	0.1	1	4
64. Agreeableness	-0.1	0.1	1	4
65. Openness to Experience	-0.2	0.1	1	4
66. Conscientiousness	-0.3	0.1	1	4
67. Neuroticism	-0.4	0.1	1	4
68. Extraversion	-0.5	0.1	1	4
69. Agreeableness	-0.6	0.1	1	4
70. Openness to Experience	-0.7	0.1	1	4
71. Conscientiousness	-0.8	0.1	1	4
72. Neuroticism	-0.9	0.1	1	4
73. Extraversion	-1.0	0.1	1	4
74. Agreeableness	-1.1	0.1	1	4
75. Openness to Experience	-1.2	0.1	1	4
76. Conscientiousness	-1.3	0.1	1	4
77. Neuroticism	-1.4	0.1	1	4
78. Extraversion	-1.5	0.1	1	4
79. Agreeableness	-1.6	0.1	1	4
80. Openness to Experience	-1.7	0.1	1	

FLEHR, HOHBACH, TEST,
ALBRITTON & HERBERT
Suite 3400, Four Embarcadero Center
San Francisco, California 94111

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Henry A. Lester

ster

[Signature]

3/15/96

3/15/96

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USA

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South Pasadena, California 91030

inventor, if any:

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Norman Davidson

3/19/96

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USA

318 East Laurel

Sierra Madre, California 91024



Paulo Kofuji

Tac on

3/15/96

1066 San Pasqual, No. 24, Pasadena, California 91106

Brazil

1066 San Pasqual, No. 24
Pasadena, California 91106

Variable	Mean	SD	Min	Max
Age	38.5	12.5	25	65
Gender	Male	100%		
Marital status	Married	100%		
Education	High school	100%		
Occupation	Teacher	100%		
Income	\$30,000	\$10,000	\$20,000	\$40,000
Health status	Good	100%		
Smoking status	Non-smoker	100%		
Alcohol consumption	None	100%		
Exercise frequency	Weekly	100%		
Stress level	Low	100%		
Sleep quality	Good	100%		
Dietary habits	Healthy	100%		
Family size	2	1	1	3
Work hours	40	5	35	45
Commuting time	30	10	20	40
Childcare costs	\$500	\$100	\$400	\$600
Health insurance	Private	100%		
Retirement savings	Yes	100%		
Debt level	Low	100%		
Life satisfaction	High	100%		
Community involvement	Active	100%		
Volunteer hours	10	5	0	20
Charitable donations	\$100	\$50	\$0	\$200
Political participation	Yes	100%		
Environmental awareness	High	100%		
Waste recycling	Yes	100%		
Energy conservation	Yes	100%		
Water conservation	Yes	100%		
Green building	Yes	100%		
Sustainable consumption	Yes	100%		
Ethical sourcing	Yes	100%		
Local food consumption	Yes	100%		
Organic food consumption	Yes	100%		
Plant-based diet	Yes	100%		
Reduced meat consumption	Yes	100%		
Minimal food waste	Yes	100%		
Composting	Yes	100%		
Reusable shopping bags	Yes	100%		
Minimal packaging	Yes	100%		
Refillable water bottles	Yes	100%		
Reusable containers	Yes	100%		
Minimal single-use plastics	Yes	100%		
Minimal fast food consumption	Yes	100%		
Minimal processed food consumption	Yes	100%		
Minimal sugary beverage consumption	Yes	100%		
Minimal alcohol consumption	Yes	100%		
Minimal tobacco consumption	Yes	100%		
Minimal gambling	Yes	100%		
Minimal pornography consumption	Yes	100%		
Minimal social media consumption	Yes	100%		
Minimal video gaming	Yes	100%		
Minimal late-night TV consumption	Yes	100%		
Minimal late-night internet browsing	Yes	100%		
Minimal late-night eating	Yes	100%		
Minimal late-night drinking	Yes	100%		
Minimal late-night smoking	Yes	100%		
Minimal late-night gambling	Yes	100%		
Minimal late-night pornography consumption	Yes	100%		
Minimal late-night social media consumption	Yes	100%		
Minimal late-night video gaming	Yes	100%		
Minimal late-night late-night TV consumption	Yes	100%		
Minimal late-night late-night internet browsing	Yes	100%		
Minimal late-night late-night eating	Yes	100%		
Minimal late-night late-night drinking	Yes	100%		
Minimal late-night late-night smoking	Yes	100%		
Minimal late-night late-night gambling	Yes	100%		
Minimal late-night late-night pornography consumption	Yes	100%		
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Minimal late-night late-night late-night late-night video gaming	Yes	100%		
Minimal late-night late-night late-night late-night late-night TV consumption	Yes	100%		
Minimal late-night late-night late-night late-night late-night internet browsing	Yes	100%		
Minimal late-night late-night late-night late-night late-night				